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13. ABSTRACT (Maximum 200 Words)
Most soy-breast cancer epidemiological studies conclude that Asian women consuming a traditional diet high in soy products have a low incidence of breast cancer. We have previously demonstrated that prepubertal exposure to genistein, the primary isoflavone of soy, protects against chemically-induced mammary cancer. The purpose of this work was to determine if adult exposure to genistein will protect against chemically-induced mammary cancer and to investigate DNA methylation of estrogen receptor genes as the molecular mechanism of genistein chemoprevention. We have determined that adult only exposure to genistein does not protect against dimethylbenz(a)anthracene (DMBA)-induced mammary cancer. However, prepubertal plus adult exposure to 250 mg genistein/kg AIN-76A diet protected against DMBA-induced mammary cancer. Prepubertal genistein exposure appears to "imprint" for additional adult genistein chemoprevention. Genistein down-regulates ER-alpha, but not ER-beta and androgen receptor, mRNA and protein expression in the rat mammary gland. The down-regulation of ER-alpha is not via DNA methylation gene silencing mechanism as measured via methylation sensitive and insensitive enzymes and Southern blots. On the other hand, genistein in the diet does up regulate overall maintenance, but not de novo, DNA methyltransferase activity, whose specificity needs to be resolved.

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Genistein, rat, breast, cancer chemoprevention

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INTRODUCTION

We have previously demonstrated that short-term exposure of rats to genistein, a soy phytoestrogen, early in postnatal life suppressed chemically-induced mammary cancer in adult rats (1, 2). This novel finding supports the epidemiological reports that Asian women consuming a traditional diet high in soy products have a low incidence of breast cancer (3-6). Furthermore, adjustment for migration rates of Asians to the U.S. revealed that the second, but not the first, generation loses this protection (6, 7). This suggests that exposure to soy early in life confers life-time protection against breast cancer. Since short-term genistein treatment early in postnatal life exerted long-term protection against chemically-induced mammary cancer, we have hypothesized that genistein caused this effect via an imprinting mechanism. For this research, we proposed to investigate 1) the potential of adult genistein treatment to alter susceptibility for breast cancer and 2) DNA methylation as the molecular mechanism of action.

BODY

Specific Aim 1) to determine the risk of mammary cancer from adult exposure to genistein. This was investigated in the rat-DMBA mammary cancer model. While this was reported in 2002, we summarize the results for the final report.

We investigated the potential of prepubertal <u>and/or</u> adult genistein exposure to protect against DMBA-induced mammary cancer. The purpose of this experiment was to determine if early critical exposure (prepubertal) to genistein would influence how the adult animal would respond to future genistein treatment. Group 1 was fed AIN-76A diet containing 250 mg genistein/kg diet, starting from parturition through day 21 only, and then AIN-76A onward (Gen/DMBA/Zero). Group 2 was fed the genistein diet from parturition through day 21, then AIN-76A only through day 100 postpartum and then from day 100, the genistein-containing diet (Gen/DMBA/Gen). Group 3 consisted of rats exposed to AIN-76A diet throughout the study (Zero/DMBA/Zero). All animals received 80 mg DMBA/kg BW at day 50. As seen in Figure 4 of reference 8 (enclosed publication), prepubertal genistein only exposure (Group 1: Gen/DMBA/Zero) provided protection against DMBA induced mammary cancer (Group 3: Zero/DMBA/Zero), confirming our previous work (1, 2). Furthermore, genistein fed to adult rats already exposed to genistein prepubertally (Group 3: Gen/DMBA/Gen) resulted in an added level of protection compared those exposed to genistein prepubertally only (Gen/DMBA/Zero).

Specific Aim 2) To investigate genistein imprinting by DNA methylation mechanism, including the potential of genistein to alter estrogen receptor gene methylation as mechanism for mammary cancer chemoprevention.

DNA methylation is considered an epigenetic mechanism whereby hypermethylation causes gene silencing and hypomethylation allows gene expression (9). There are two functional patterns of methylation, 1) *de novo* methylation which starts *in utero* and continues during early postnatal development, and 2) maintenance methylation that predominates later in life. Methylation of promoter CpG islands leads to binding of methylated CpG binding proteins and transcription repressors to block transcription initiation (10).

Measurement of DNA methyltransferase activity, with non-methylated oligonucleotide template was used as an indicator of *de novo* methylation, and hemi-methylated DNA (a 60-mer

oligonucleotide template containing CpG sequence) was used for analysis of maintenance methylation. Analyses revealed that *de novo* and maintenance DNA methylation activities in rat mammary glands were higher at 21 and 50 days than at 124 days (Figure 1).

Measurement of DNA methylation activities in mammary glands of 21 day old rats exposed to dietary genistein from parturition to the time of weaning (day 21) demonstrated a significant increase in maintenance-, but not in *de novo*-, DNA methylation activity (Figure 1A). Measurement of DNA methylation activities in mammary glands of 50 day old rats exposed to dietary genistein from birth to day 21 only did not reveal any change in DNA methylation activities (Figure 1B). However, maintenance methylation activity was significantly higher in mammary glands of 50 day old rats exposed to genistein from birth until day 50 (Figure 1C), suggesting that genistein is a direct modulator of DNA methylation activity (Figure 1A and Figure 1C) and does not permanently increase DNA methylation activity (Figure 1B). Interestingly neither *de novo*-, or maintenance-, DNA methylation activity was modulated in mammary glands of 124 day old rats treated with genistein from birth until 21 days old only (Figure 1D) or from 100-124 days postpartum only (Figure 1E). On the other hand maintenance, but not *de novo*-, DNA methylation activity was increased in mammary glands of 124 day old rats exposed to genistein from parturition to 21 days, and then from 100 to 124 days (Figure 1F). This suggest that early postnatal exposure to genistein is necessary for adult response to the same challenge for maintenance DNA methylation activity. In rodents, imprinting appears to occur during the perinatal period, resulting in permanent manifestations that can occur even in the absence of the original effector (11).

There are 3 main families of DNA (cytosine-5') methyltransferases (Dnmts), 1-3. The ontogeny of the Dnmt 3 family implicates the proteins as being essential for *de novo* methylation which is methylation of previously unmethylated DNA. The catalytic domain of DMNT 2 lacks catalytic activity. Maintenance methylation is dependent on a methyltransferase, DNMT 1, which can recognize the normally methylated CpG sites in the parent strand and catalyze addition of a methyl group to the cytosine in the corresponding CpG site of the daughter strand. However, under certain circumstances, DNMT1 may also promote *de novo* CpG methylation (12, 13). To determine if DNMT protein levels were increased, we used western blot analysis for quantitation. In mammary glands of 21 day old rats exposed from 1-21 days postpartum to genistein we found similar levels of DNMT 1, 3a and 3b proteins (Figure 2). Likewise, genistein in the diet from 1-50 days did not alter DNMT 1, 3a and 3b protein levels (Figure 2). This demonstrates that genistein is directly modulating DNA methyltransferase activity (Figure 1) and not simply increasing the amount of DNMT protein(s).

Because of genistein's phytoestrogen action, we investigated estrogen receptor (ER) expression by western blot analysis. Mammary glands of 50 day old female rats exposed to dietary genistein from 1-50 days postpartum had reduced expression of ER-alpha protein (Figure 3). However, no change in ER-alpha was observed in 50-day old rats exposed to dietary genistein from birth through 21 days only (data not shown). This suggest that the action of genistein on ER-alpha is direct. Also, we measured ER-beta receptor and androgen receptor expressions by western blot analysis and did not find changes in their expressions (data not shown).

To determine if the decrease in ER-alpha protein was due to reduced RNA transcripts, we measured mRNA levels using reverse transcriptase-polymerase chain reaction, and found decreased mRNA levels of ER-alpha in mammary glands of 50 day old rats (Figure 4). To

determine if the decreases in ER-alpha protein and mRNA levels were due to gene silencing, we investigated DNA methylation of the ER-alpha gene promoter. Using Hmn as flanking enzyme and Ava and HhaI as methylation-sensitive enzymes, no change in DNA methylation status was observed in mammary glands of rats exposed to genistein since birth compared to control (Figure 5). Similar results (no change in methylation) were obtained with HhaI as methylation sensitive enzyme. Likewise, no change in DNA methylation of the ER-alpha promoter was observed in 21 and 124 day old rats exposed to genistein. DNA methylation of ER-beta and androgen receptor was not investigated because the protein and mRNA levels of these two sex steroid receptors were not regulated by genistein. We conclude that genistein does not alter DNA methylation of ER-alpha, ER-beta and androgen receptors.

Other Completed Tasks. The P.I. has attended and made presentations at the AACR meetings in 2001-2003 and the 2002 DOD Era of Hope Breast Cancer Meeting in Orlando. Evidence of these are presented in Reported Outcomes. Manuscripts have been published and a 3rd manuscript related to the findings of Specific Aim 2 is being prepared.

KEY RESEARCH ACCOMPLISHMENTS

1) Dietary genistein given to adult female rats after tumors were initiated did not alter the multiplicity of mammary tumors. This can be interpreted as genistein not exerting a chemotherapeutic effect on existing tumors, and genistein not exacerbating development of previously existing mammary tumors.

2) On the other hand, dietary genistein to adult rats exposed prepubertally to genistein provides additional protection against mammary cancer. Prepubertal genistein exposure appears to "imprint" for additional adult genistein chemoprevention.

3) Exposure to genistein in the diet (and via mother's milk) increases DNA methyltransferase activity, but not the amount of DNMT proteins as measured by western blot analysis. In 124 day old rats, adult regulation of DNA methyltransferase activity is dependent on early postnatal exposure to genistein.

4) ER-alpha, but not ER-beta and AR, mRNA and protein expressions are decreased in mammary glands of 50 day old rats treated with genistein. Measurements of DNA methylation of the ER-alpha gene promoter suggest that gene silencing does not account for the reduced expression of ER-alpha protein. Others have reported that response of the uterus to estrogen stimulation was characterized by decreased ER protein (14), suggesting protein turnover to maintain homeostasis.

REPORTABLE OUTCOMES

Publications
Lamartiniere, C.A., Cotroneo, M.S., Fritz, W.A., Wang, J. Roycelynn Mentor-Marcel, R.-M. and Ada Elgavish, A. Genistein Chemoprevention: Timing and Mechanisms of Action in Murine Mammary and Prostate. J. Nutrition. 132: 552S-558S, 2002.

Cotroneo, M.S. Wang, J., Fritz, WA, Eltoum, I.-E. and Lamartiniere, C.A. Genistein action in the prepuberatal mammary gland in a chemoprevention model. Carcinogenesis. 23: 1467-1474,

2002.

Presentations
<u>Fourth International Symposium on the Role of Soy in Preventing and Treating Chronic Disease</u>:
Dietary Genistein Protects Against Mammary and Prostate Cancers. San Diego, CA. Nov 4-7,
2001.

<u>Roche Vitamins and Fine Chemicals.</u> Dietary Genistein Protects Against Mammary and Prostate
Cancers. Basel Switzerland. July 5, 2002.

<u>Era of Hope Breast Cancer Meeting (DOD)</u>. Symposium Presenter: Genistein Programming
Against Breast Cancer. Orlando FL. Sept. 25-28, 2002.

CONCLUSIONS

We conclude that dietary genistein in adult life is only effective in protecting against chemically-
induced mammary cancer if the female mammary gland has already been imprinted
prepubertally. Genistein down-regulates ER-alpha, but not ER-beta and androgen receptor,
mRNA and protein expressions in the rat mammary gland. The down-regulation of ER-alpha is
not *via* DNA methylation gene silencing mechanism. On the other hand, genistein in the diet
does up regulate overall maintenance DNA methylation activity, whose specificity needs to be
resolved.

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    R.-M. and Ada Elgavish, A. Genistein Chemoprevention: Timing and Mechanisms of
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B.J. Patel
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APPENDICES
5 Figures
2 Publications

Figure 1. Genistein Regulation of DNA Methyltransferase Activity in Rat Mammary Glands
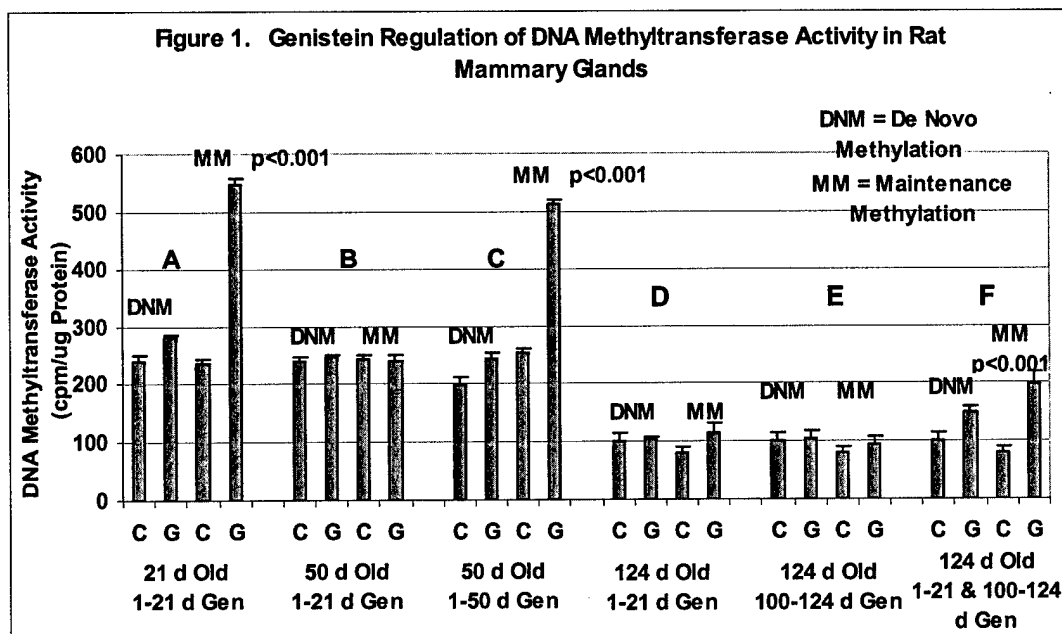
Experiment A: Rats were exposed to genistein from birth until 21 days old to 250 mg genistein/kg AIN-76A diet (G) or control AIN-76A diet (C) and killed at day 21. Experiment B: Rats were exposed to genistein from birth until 21 days only to genistein diet and killed at day 50. Experiment C: Rats were exposed to genistein from birth until 50 days to genistein diet and killed at day 50. Experiment D: Rats were exposed to genistein from birth until 21 days only to genistein diet and killed at day 124. Experiment E: Rats were exposed to genistein from days 100-124 to genistein diet or control diet and killed at day 124. Experiment F: Rats were exposed to genistein from birth until 21 days and from 100-124 days to genistein diet and killed at day 124. Average values for each treatment group were compared to respective control group and methylation activity using one-way of variance (ANOVA) and reported as percent of control.

Figure 2. Western Blot Analysis for DNA Methyltransferases in Mammary Glands of 21 and 50 Day Old Rats

C: Control AIN-76A diet; G: 250 mg genistein/kg AIN-76A diet
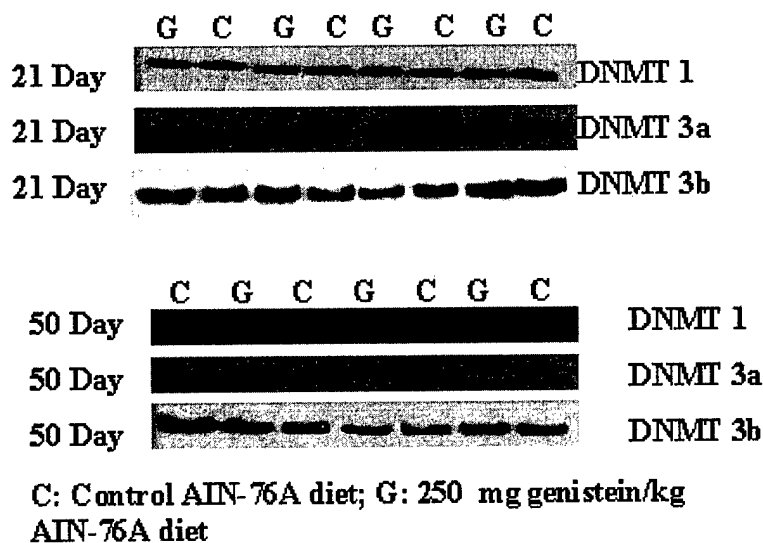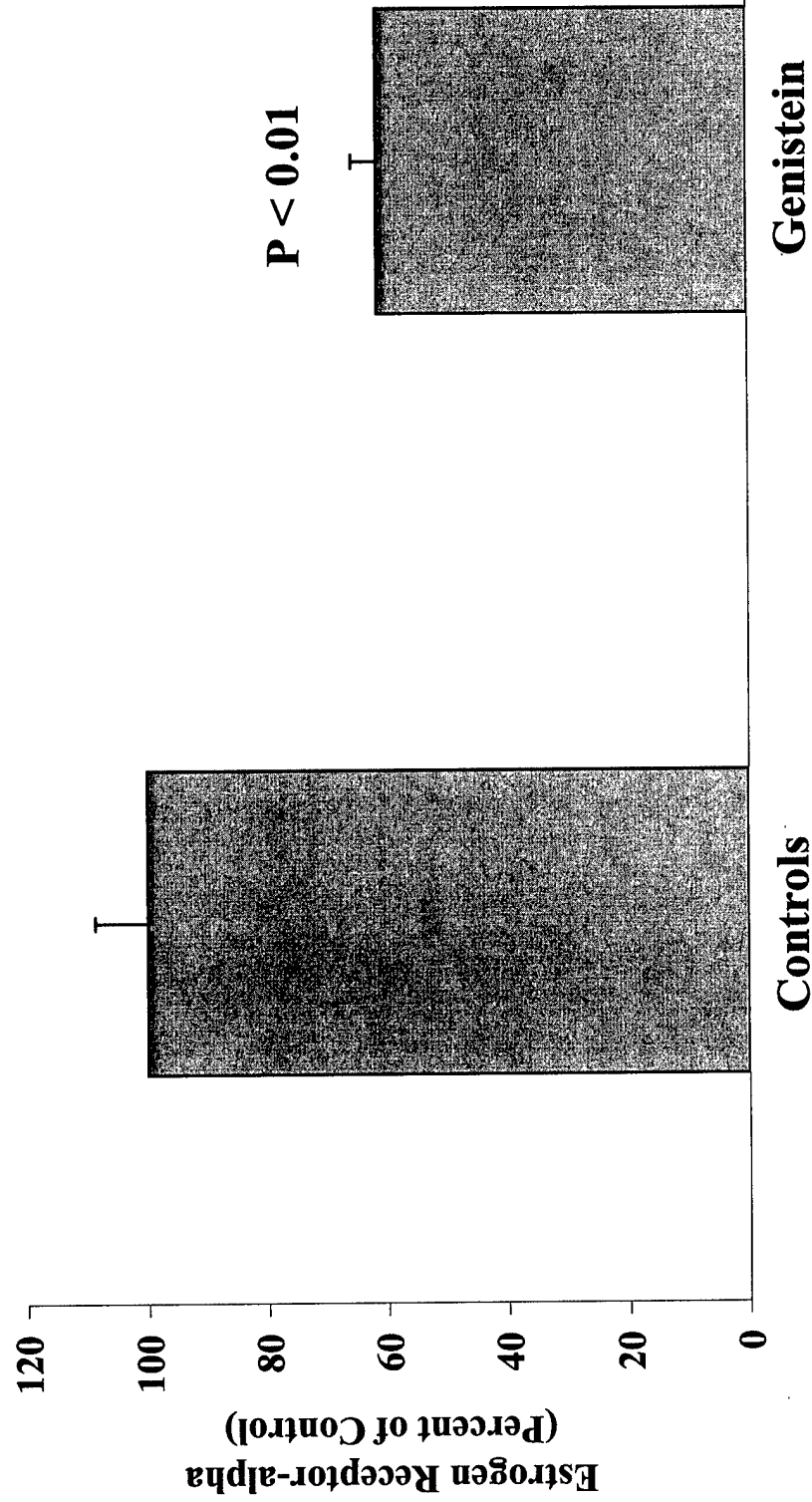
Figure 3. Genistein regulation of ER-alpha in mammary glands of 50 day old rats exposed to genistein from birth until 50 days.

C G C G C G C G C G C G C G C ER-alpha

Estrogen Receptor-alpha (Percent of Control)

120 100 80 60 40 20 0

P < 0.01

Controls   Genistein

C: Control AIN-76A diet; G: 250 mg genistein/kg AIN-76A diet

Figure 4. Estrogen receptor-alpha mRNA levels in mammary glands of 50 day old rats exposed to genistein from birth until 50 days.

MW C C C C C C G G G G G G

ER-alpha mRNA Levels
(Percent of Controls)

P < 0.01

Controls

Genistein

120 100 80 60 40 20 0

MW: molecular weight standards; C: Control AIN-76A diet;
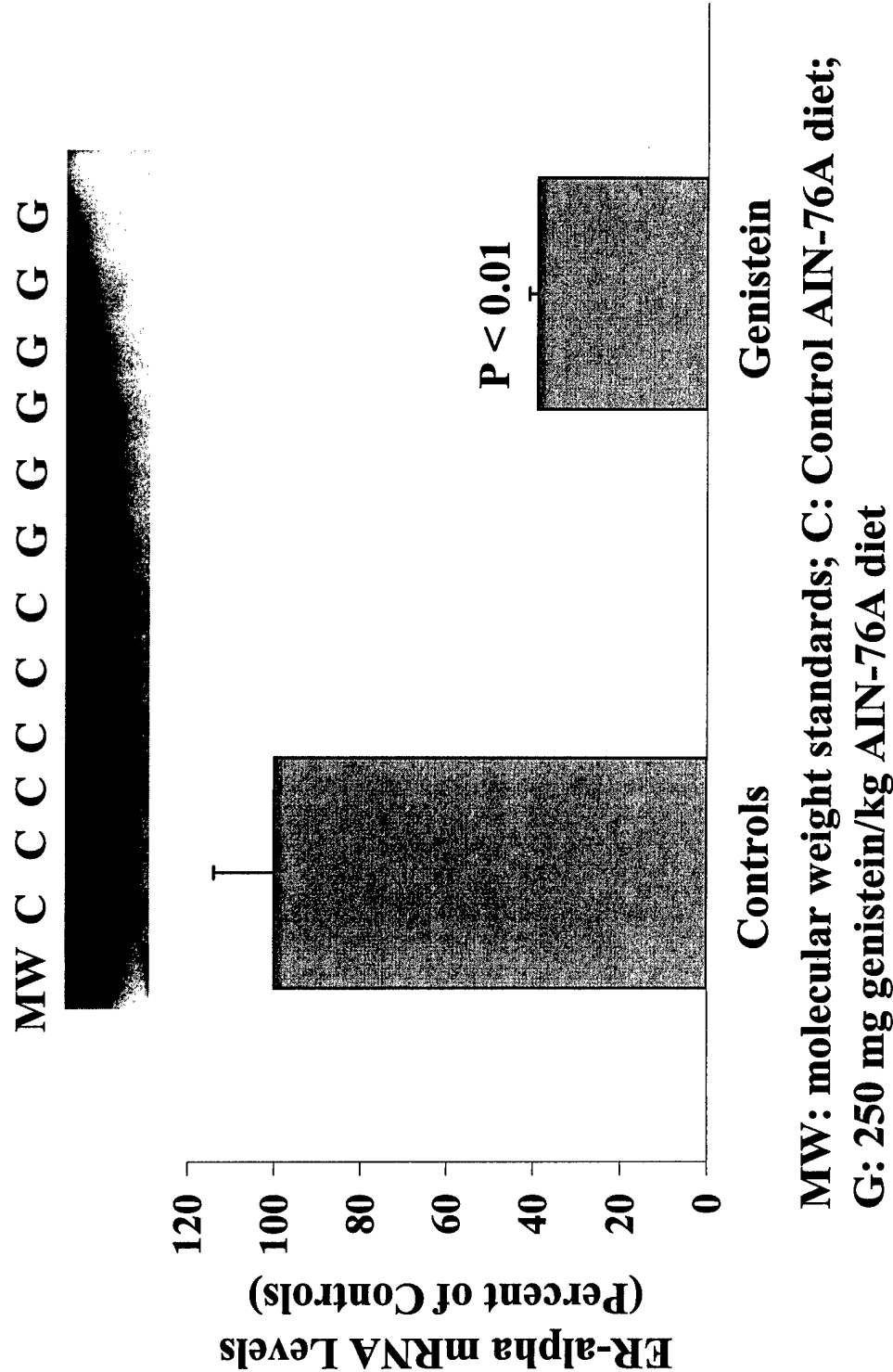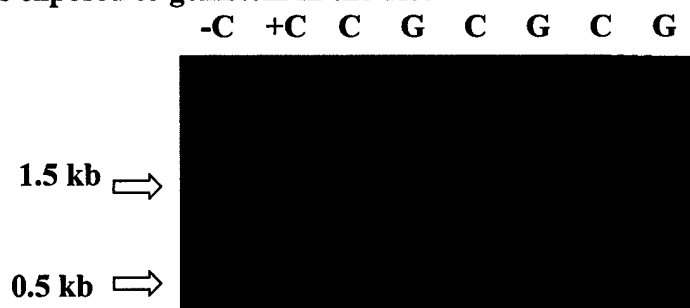G: 250 mg genistein/kg AIN-76A diet

Figure 5. Southern blot analysis for DNA methylation of ER-alpha promoter in mammary glands of rats exposed to genistein in the diet

Rats were exposed to genistein or control diet starting at birth. As controls, total genomic DNA from prostate tissue was incubated with flanking enzyme HmnI as negative control (-C), and with flanking enzyme HmnI + methylation sensitive enzyme Ava as positive control (+C). DNA from mammary tissues exposed to genistein (G) or control AIN-76A diet (C) were subjected to HmnI + Ava enzymes. Please note no DNA digest at 0.5 kb in mammary tissues indicating no change in methylation as opposed to the 0.5 kb DNA fragment in lane 2 demonstrating unmethylated DNA in control prostate sample. The ER-alpha fragment was confirmed with ^{32}P-labeled probe. The large 1.5 Kb DNA digest is from the flanking enzyme HmnI. Similar results were obtained with HhaI as methylation sensitive enzyme.

Genistein Chemoprevention: Timing and Mechanisms of Action in Murine Mammary and Prostate[1,2]

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ABSTRACT   We investigated the potential of genistein, the primary isoflavone of soy, to protect against breast and prostate cancers in animal models. For mammary cancer studies, Sprague-Dawley rats were fed AIN-76A diet ± 250 mg genistein/kg diet. Dimethylbenz[a]anthracene was administered by gavage at d 50 postpartum to induce mammary tumors. Mammary cancer chemoprevention was demonstrated after prepubertal and combined prepubertal and adult genistein treatments but not after prenatal- or adult-only treatments, demonstrating that the timing of exposure to genistein is important for mammary cancer chemoprevention. The cellular mechanism of action was found to be mammary gland and cell differentiation, as shown by whole-mount analysis and β-casein expression. An imprinting effect was shown for epidermal growth factor receptor expression in mammary terminal end buds. For prostate cancer studies, we used two models. The first was a chemically (N-methylnitrosourea) induced prostate cancer rat model. Genistein in the diet inhibited the development of invasive adenocarcinomas in a dose-dependent manner. The second model was a transgenic mouse model that resulted in spontaneously developing adenocarcinoma tumor of the prostate. Genistein in the diet reduced the incidence of poorly differentiated prostatic adenocarcinomas in a dose-dependent manner and down-regulated androgen receptor, estrogen receptor-α, progesterone receptor, epidermal growth factor receptor, insulin-like growth factor-I, and extracellular signal-regulated kinase-1 but not estrogen receptor-β and transforming growth factor-α mRNA expressions. We conclude that dietary genistein protects against mammary and prostate cancers by regulating specific sex steroid receptors and growth factor signaling pathways.   J. Nutr. 132: 552S–558S, 2002.

KEY WORDS: • genistein • chemoprevention • mammary • prostate • cancer

Cancer is usually treated at the time of diagnosis, and chemoprevention is not usually considered until adulthood. However, because perinatal exposure to hormones and xenobiotics influences breast and prostate development and cancer, we have hypothesized that exposure to hormonally active nutritional chemicals during early windows of development plays a key role for cancer causation and prevention in these tissues.

The most convincing evidence indicating that environmental agents and early periods of development predispose for breast cancer is radiation exposure. Women exposed as teenagers to ionizing radiation are more susceptible for breast cancer than those exposed as adults (1,2). Moreover, early pregnancy or early exposure to the hormones of pregnancy reduces the incidence of breast/mammary cancer in women and animal models (3–5). This demonstrates that the early period of a woman's life is crucial for predisposition to or for protection against breast cancer.

Asian women consuming a diet high in soy products have a low incidence of breast cancer (6,7), yet Asians who immigrate to the United States and adopt a Western diet lose this protection. Soy-based diets are high in phytochemicals and quantitative results indicate that isoflavone phytoestrogens are normal constituents of human urine from subjects consuming large amounts of soy products (tofu, soy flour, soy milk, tempeh, etc.) (8). Genistein is the predominant isoflavone phytoestrogen found in soy.
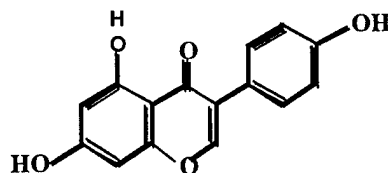
FIGURE 1 Structure of genistein.

Genistein is a planar molecule with an aromatic A-ring, has a second oxygen atom 11.5 Å from the one in the A ring, and has a molecular weight similar to those of the steroidal estrogens (Fig. 1). It has estrogenic properties in receptor binding assays (9,10), cell culture (11,12), and uterine weight assays (13–15). Genistein inhibits topoisomerase II (16), platelet-activating factor- and epidermal growth factor-induced expression of c-fos (17), diacylglycerol synthesis (18), and tyrosine kinases (19). It also inhibits microsomal lipid peroxidation (20) and angiogenesis (21). Genistein exhibits antioxidant properties (22–24) and was reported to induce differentiation of numerous cell types (25–27). Most of these mechanistic data were derived from in vitro studies.

Genistein and mammary cancer

To investigate the potential of perinatal genistein exposure to protect against chemically induced mammary cancer, female Sprague-Dawley rats were fed 0, 25 and 250 mg genistein/kg AIN-76A diet starting 2 wk before breeding (28). Animal care and treatments were conducted in accordance with established guidelines and protocols approved by the University of Alabama at Birmingham Animal Care Committee. The dietary concentrations were chosen because they yield serum genistein concentrations in rats similar to blood genistein concentrations in men and women eating a diet high in soy (28,29). After parturition, dams and offspring were fed the same diets until time of weaning (d 21). From that time onward, all female offspring from the three treatment groups were fed AIN-76A diet only. At d 50 postpartum, dimethylbenz[a]anthracene (DMBA)[4] (80 mg/kg body) was administered by gavage to induce mammary tumors. Animals were palpated for tumors and necropsied at 180 d after DMBA treatment or when tumors developed to 2.5 cm in diameter. Control animals (zero genistein, DMBA) developed approximately nine tumors, whereas dietary genistein suppressed DMBA-induced mammary tumor development in a dose-dependent manner. Rats exposed to 25 and 250 mg genistein/kg AIN-76A diets had 7.1 and 4.3 mammary tumors, respectively (Fig. 2). This dietary genistein chemoprevention study is consistent with our previous work demonstrating that injections of pharmacologic doses of genistein during the neonatal and prepubertal periods suppressed chemically induced mammary tumor development (30,31). Recently, Hilakivi-Clarke et al. (32) confirmed that prepubertal exposure to genistein reduces mammary tumorigenesis.

In the next study, we addressed the possibility that genistein exposure, via the dam during the prenatal period only, might alter the female offspring's susceptibility for mammary cancer. One group of female rats was fed 250 mg genistein/kg AIN-76A diet during breeding and pregnancy. At parturition, the dams and offspring were switched to AIN-76A
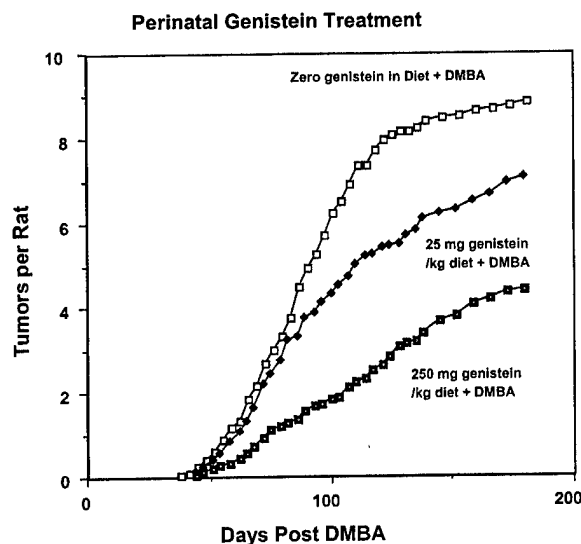
FIGURE 2 Ontogeny of palpable mammary tumors in female Sprague-Dawley CD rats exposed perinatally to genistein in the diet. Starting at time of breeding, dams were fed 0, 25 and 250 mg genistein/kg AIN-76A diet. After weaning, the offspring were fed AIN-76A diet only. On d 50 postpartum, female offspring were treated with 80 mg dimethylbenz[a]anthracene (DMBA)/kg body. [Modified from (28). Permission requested from Oxford University Press.]

diet without genistein supplement. The control group for this experiment was made up of females fed AIN-76A diet from the time of breeding throughout the experiment. Dietary exposure to genistein, merely during the prenatal period, neither increased mammary carcinogenesis nor conferred protection against DMBA-induced (80 mg/kg body) mammary cancer (Fig. 3). These data confirm our previous prenatal genistein study using a lower dose of DMBA (40 mg/kg body) (29). Data from Figures 2 and 3 show that the critical window for genistein chemoprevention is the postnatal time of the perinatal period.
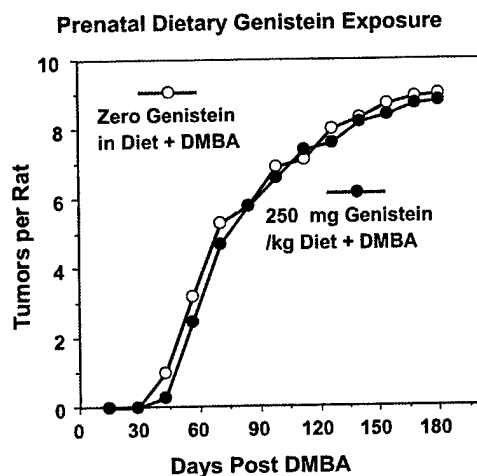

FIGURE 3 Ontogeny of palpable mammary tumors in female offspring of Sprague-Dawley CD rats fed genistein in the diet during pregnancy. Two groups of pregnant female rats (25 each) were fed 0 or 250 mg genistein/kg AIN-76A diet. At parturition both groups were fed AIN-76A diet until the time of necropsy (230 d postpartum). All offspring were treated with 80 mg DMBA/kg body on d 50 postpartum.
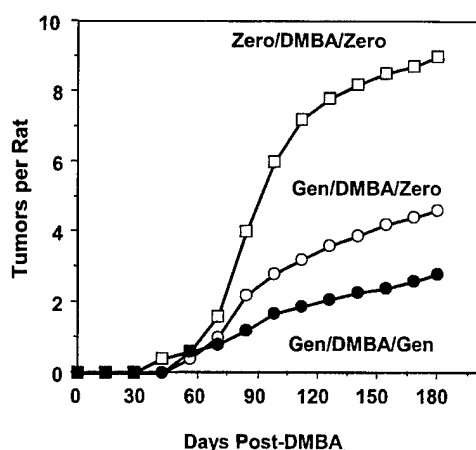
FIGURE 4 Adult dietary genistein effect on palpable mammary tumors in rats exposed prepubertally to genistein and as adults to DMBA. Group 1 was fed control AIN-76A diet starting from parturition and continued throughout the study (Zero/DMBA/Zero). Group 2 was fed AIN-76A diet containing 250 mg genistein/kg diet, starting from parturition through d 21 only and then AIN-76A onward (Gen/DMBA/Zero). Group 3 was fed genistein-containing diet from parturition through d 21, AIN-76A only through d 100 postpartum, and genistein-containing diet (Gen/DMBA/Gen) from d 100. All rats received 80 mg DMBA/kg body at d 50. Each group consisted of 25 rats.

Our data (Fig. 3) demonstrate that dietary genistein administered prenatally did not alter predisposition for mammary cancer. In contrast, Hilakivi-Clarke et al. (33) reported that injecting pregnant rats with genistein resulted in increased susceptibility of the offspring for mammary cancer. We speculated that this apparent contradiction might be due to different routes of administration and bioavailability in the two studies. Circulating genistein concentrations from 21-d fetal, 7-d neonatal and 21-d prepubertal rats exposed to 250 mg genistein/kg AIN-76A diet were determined to be 43, 726 and 1810 nmol/L, respectively (28,29). This demonstrates genistein bioavailability during postnatal life but poor bioavailability prenatally. Also, we determined that ~46% of circulating total genistein is free genistein 24 h after injection of rats (34). This is in contrast to <2% being free (aglycone) genistein from dietary administration (28). The bioavailability of injected genistein is substantially greater than that of oral genistein (23-fold). Hence, we conclude that route of administration and timing of exposure determines the metabolism, bioavailability and biological action of genistein.

Because breast cancer has been demonstrated to be estrogen-dependent, we have been concerned that genistein, a phytoestrogen, may contribute to mammary cancer development. More specifically, women who have been diagnosed with breast cancer inquire whether soy products, including genistein, will protect from or cause a recurrence of their cancer. We attempted to address this in a laboratory study. Rats were fed AIN-76A diet ± 250 mg genistein/kg diet at three periods, and all females were treated intragastrically with 80 mg DMBA/kg body. As seen in **Figure 4**, rats exposed to the control diet, AIN-76A only, from birth until the end of the experiment (Zero/DMBA/Zero) had the highest average number of tumors (9.0 tumors/rat). Rats exposed to genistein from d 1 to 21 postpartum only (Gen/DMBA/Zero) developed 4.5 tumors, which confirms our earlier work (28). Animals exposed to genistein from d 1 to 21 and 100 to 180 (Gen/DMBA/Gen) developed the fewest number of tumors (2.8 tumors/rat). The latter genistein feeding was initiated 50 d

after the DMBA treatment, the time of onset of palpable mammary tumors. This demonstrates that genistein fed to adult rats previously exposed prepubertally to genistein provided these rats with additional protection against mammary cancer. Prepubertal genistein exposure seems to permanently affect the animal or mammary gland in a way that determines how that animal later responds to the same or similar chemical stimuli. In this case, genistein fed during the prepubertal period programmed future (adult) genistein response against mammary cancer susceptibility.

Table 1 summarizes the relationship among dietary genistein, timing of exposure and chemically induced mammary cancer in rats. Limiting exposure to dietary genistein to the prenatal or adult periods does not predispose or protect against mammary cancer. In contrast, exposure to dietary genistein during the prepubertal and prepubertal-plus-adult periods protected against chemically induced mammary cancer in rats. An epidemiological report using the Shanghai Cancer Registry, a case-control study, has shown an inverse relationship (50%) between adolescent (13–15 y old) soyfood intake and breast cancer incidence later in life (35).

Genistein mechanism of action in the mammary gland

Analysis of mammary gland morphology in rats treated with genistein revealed that its cellular mechanism of action is enhancement of mammary gland differentiation (28–31). We demonstrated that genistein administered to prepubertal rats reduced the number of terminal end buds and increased the number of lobules. Mammary terminal end buds are terminal ductal structures found primarily in young animals (and humans) and contain many undifferentiated epithelial cells (36,37). Terminal end buds are the most susceptible structures to chemical carcinogens; lobules are the terminal ductal structures most differentiated and least susceptible to chemical carcinogens.

Further evidence that genistein enhances differentiation was obtained by measuring β-casein in mammary glands. β-casein is a milk protein and biomarker of mature mammary glands and differentiated cells. Using Western blot analysis, we found that prepubertal genistein treatment increased β-casein expression in mammary glands of prepubertal and adult rats (**Fig. 5**). In the adult rats, β-casein was measured 30 d after genistein treatment.

One of the reasons cancer researchers have investigated

TABLE 1

Dietary genistein, timing of exposure and mammary cancer chemoprevention

Exposure period	Relative mammary tumor multiplicity[1]
No genistein	8.9
Prenatal genistein[2]	8.8
Adult genistein (after tumors)[3]	8.2
Prepubertal genistein[4]	4.3
Prepubertal and adult genistein[3,4]	2.8

Diets contained ± 250 mg genistein/kg AIN-76A.
[1] All rats were treated with 80 mg dimethylbenz[a]anthracene/kg body weight at d 50 postpartum.
[2] Prenatal treatment is throughout gestation.
[3] Adult treatment was initiated at 100 d postpartum.
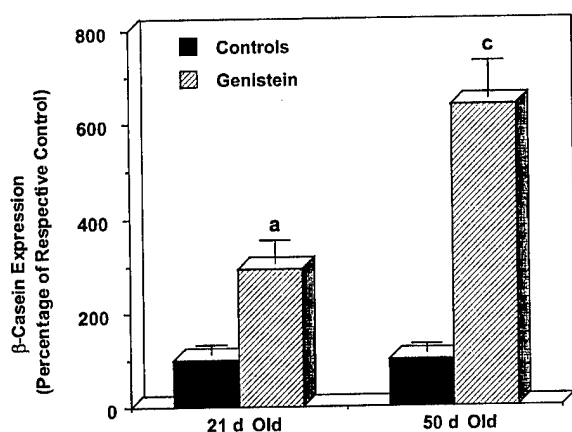[4] Prepubertal treatment was from d 1 to 21 postpartum.

FIGURE 5 β-casein expression in mammary glands of rats treated prepubertally with genistein. Female Sprague-Dawley CD rats were injected subcutaneously with 500 μg genistein/g body or an equivalent volume of the vehicle, dimethylsulfoxide, on d 16, 18 and 20 postpartum. Western blot analysis was carried out to measure β-casein expression in mammary glands of 21- and 50-d-old rats (six animals per group). The β-casein antibody was a generous gift from Margaret Benton and Michael Gould of the University of Wisconsin, Madison.

genistein as a chemopreventive agent is the reports that it inhibits protein tyrosine kinases in vitro (18,19). As an extension of this, we investigated the potential of genistein to regulate the epidermal growth factor (EGF) receptor in vivo. In 21-d-old rats treated prepubertally with genistein, we found increased EGF receptor expression in mammary terminal end buds (38). Not only was this finding contrary to the in vitro reports, but this was surprising because we expected a chemopreventive agent to down-regulate the expression of this growth factor signaling pathway. However, when we extended our studies to 50-d-old rats treated prepubertally with genistein, we observed that EGF receptor expression was down-regulated in terminal end buds of these rats. We interpreted this to mean that early in postnatal life, genistein initially up-regulated the EGF-signaling pathway to enhance mammary gland development that resulted in early mammary gland differentiation. Reduced EGF signaling and decreased cell proliferation at 50 d when the DMBA was given was associated with reduced susceptibility to chemical carcinogenesis (28–31,36,37). Developmental modifications by a hormonally active chemical that result in altered biochemical or behavioral responses later in life was defined as imprinting (39–41). We speculate that down-regulated EGF receptor signaling in mammary terminal end buds at the time of carcinogen exposure plays a role in reduced mammary cancer development.

Genistein and mammary cancer chemoprevention summary

We have demonstrated that prepubertal and prepubertal-plus-adult genistein exposures protect against chemically induced mammary cancer in rats. We conclude that for genistein to protect against breast cancer, initial exposure must occur during the early sensitive period of mammary gland development, that is, the neonatal through prepubertal periods. The cellular mechanism of action of genistein is to enhance mammary cell differentiation (28–31). One identified biochemical mechanism is short-term and direct up-regulation of the EGF-signaling pathway that plays a role in cell differentiation (38). Paradoxically, this results in the epithelial cells of the mam-

mary terminal end buds of adult animals having reduced EGF receptor expression. EGF signaling has been associated with cell proliferation; hence, we believe that down-regulated EGF receptor in adults contributes to the genistein chemoprevention. In reference to genistein in adults conferring additional protection when given to rats previously exposed to genistein compared with genistein-naive animals, we speculate that the early developmental effects have altered the molecular blueprint from which mammary cells respond to cancer initiators and promoters. Our laboratory data are consistent with the epidemiological report showing an inverse relationship between adolescent soyfood intake and breast cancer incidence later in life (35). We conclude that the most sensitive period for mammary cancer chemoprevention in the rat is the prepubertal period and in the human is probably the adolescent period.

Genistein and prostate cancer

Prostate cancer is the second leading cause of cancer death in men. Epidemiological data indicate that the incidence and mortality of prostate cancer are considerably lower in Asian populations than in U. S. and European populations (42), yet the incidence of precancerous lesions is the same for these populations (43). Upon emigration to the United States, Asian men have a greater risk for developing prostate cancer, and the earlier in life their arrival, the more closely their risk approaches that of American men (44).

One of the major differences between Asian and Western populations is diet. Asians have traditionally consumed a soy-based diet containing isoflavones, resulting in higher genistein concentrations in the blood and urine than those of American men (45,46). Our goal was to investigate the potential of genistein in the diet to protect against prostate cancer.

For the first prostate chemoprevention study, Lobund-Wistar rats were exposed to 0, 25 and 250 mg genistein/kg AIN-76A diet starting at conception and continuing until necropsy at age 11 mo (47). From d 50 to 66 postpartum, male offspring were given 33 mg flutamide/kg body daily by gavage to cause chemical castration. On d 67, 68 and 69, they were injected daily with 25 mg testosterone/kg body to stimulate mitosis. On d 70, all rats were anesthetized and 42 mg N-methylnitrosourea (MNU)/kg body was injected into the dorsal prostate for cancer initiation. One week after MNU administration, silastic implants of 25 mg testosterone were administered (and replaced every 12 wk) to stimulate mitosis and promote tumor growth. By age 40 wk, palpable prostate tumors were detectable. Rats were necropsied when 48 wk old or when they became moribund. In rats with small tumors, the tumors were confined to the site of MNU injection, demonstrating target organ specificity.

Rats fed the control diet, AIN-76A, and subjected to the carcinogenesis protocol developed 86.4% incidence of prostate tumors by 11 mo old (**Fig. 6**). Rats exposed to 25 and 250 mg genistein/kg diet had tumor incidences of 77.8% and 63.0%, respectively. The percentage of prostate tumors that were classified as invasive adenocarcinomas in rats fed 0, 25 and 250 mg genistein/kg diet were 77.3%, 61.1% and 44.4%, respectively. This was a dose-dependent significant decrease in prostate adenocarcinoma development (47). We conclude that lifetime dietary genistein protected against chemically induced prostate cancer development in rats.

The second model used for investigating genistein chemoprevention of prostate cancer was a transgenic mouse model that spontaneously develops prostate cancer, transgenic mouse
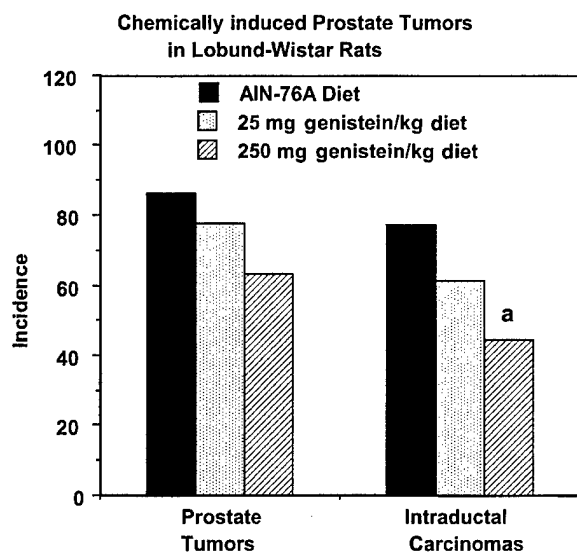
Chemically induced Prostate Tumors in Lobund-Wistar Rats

FIGURE 6 Prostate cancer incidence in Lobund-Wistar rats fed genistein in the diet. Lobund-Wistar rats were provided 0, 25 and 250 mg genistein/kg AIN-76A diet starting at conception. Male offspring were treated with 33 mg flutamide/kg body by gavage on d 50–66 and injected with 25 mg testosterone/kg on d 67–69; 42 mg methylnitrosourea/kg injected into the dorsolateral prostate on d 70; and 25-mg testosterone implants were started on d 77 (and replaced every 12 wk). Animals were necropsied when 48 wk old or when moribund. [a]$P = 0.04$ compared with the AIN-76A diet group (Fisher exact test), and $P = 0.03$ by Cochran-Armitage trend test for tumor invasive adenocarcinomas. [Data modified from (47). Permission granted from Elsevier Science Ireland.]

prostate adenocarcinoma (TRAMP) (48). The TRAMP mouse was developed by using the prostate-specific probasin promoter to drive expression of the simian virus 40 early gene in the prostatic epithelium. The SV40 T antigen (Tag) acts as an oncoprotein through interactions with the p53 and retinoblastoma tumor-suppressor gene products. All TRAMP mice develop changes resembling human prostate intraepithelial neoplasia and poorly differentiated tumors, ultimately developing prostatic adenocarcinomas that metastasize to distant sites, primarily the lymph nodes, bone and lungs (49,50).

In our experiments (51), approximately one-half of the transgenic male mice displayed well-differentiated prostatic adenocarcinoma by 28 wk old; the other one-half was divided between moderately differentiated and poorly differentiated adenocarcinomas. To test the potential of genistein to prevent poorly differentiated adenocarcinomas, transgenic males were fed 0, 100, 250 or 500 mg genistein/kg AIN-76A diet, starting at 5–6 wk old. Mice remained on the diet until they were 28–30 wk old. The proportion of transgenic males that developed poorly differentiated adenocarcinoma was significantly reduced in a dose-dependent manner by dietary genistein (**Fig. 7**).

At necropsy, serum genistein concentration was determined and selected organs were weighed and prepared for histopathological evaluation (51). Serum genistein concentrations in mice on diets containing 0, 250 or 500 mg genistein/kg AIN-76A were 52 ± 33, 139 ± 70 and 397 ± 105 nmol/L, respectively, comparable with those found in Asian men on a regular soy diet (276 nmol/L) (45). As indicated by body and organ weights, dietary genistein had no toxic effect on TRAMP mice.

Sex steroid and growth factor signaling in the prostate

The interaction of sex steroid and growth factor signaling pathways is believed to be critical in the process of development and differentiation of hormone-responsive tissues and for cancer in the prostate (52). However, whether steroid hormones mediate the effects of growth factors or vice versa is unclear. Sex steroid-induced epithelial cell proliferation and differentiation have been associated with the coordinated induction of several peptide growth factors and their receptors, including some that are tyrosine kinase-dependent.

Nontransgenic and TRAMP mice were fed AIN-76A diet until 6 wk old, when one group of 14 TRAMP mice was fed 250 mg genistein/kg diet. An equal number of TRAMP and nontransgenic mice were fed AIN-76A diet only. At 12 wk old, the three groups of mice were killed and the dorsolateral prostates were collected. This is the period of prostate intraepithelial neoplasia and preneoplastic development in the prostate of TRAMP mice but before development of adenocarcinoma tumor (48,53,54). RNA was isolated and reverse-transcribed and the cDNA was amplified by polymerase chain reaction. Relative quantitative differences in cDNA were determined from data obtained during the exponential phase of amplification. In comparing prostates of transgenic and nontransgenic mice, we observed that androgen receptor, estrogen receptors (ER-α and -β), progesterone receptor, EGF receptor, transforming growth factor-α, insulin-like growth factor I and extracellular regulating kinase-1 mRNA transcripts were significantly higher in the transgenic mice (C. A. Lamartiniere and J. Wang, unpublished data, 2001). We speculate that increased sex steroid and growth factor signaling contribute to the increased incidence of spontaneously developing prostate cancer in transgenic mice.

In contrast, the prostates of transgenic mice fed the genistein-containing diet had reduced androgen receptor, ER-α, progesterone receptor, EGF-receptor, insulin-like growth factor I and extracellular regulating kinase-1 mRNA transcripts compared with prostates from TRAMP mice fed a

Spontaneously Developing Prostate Tumors in TRAMP Mice
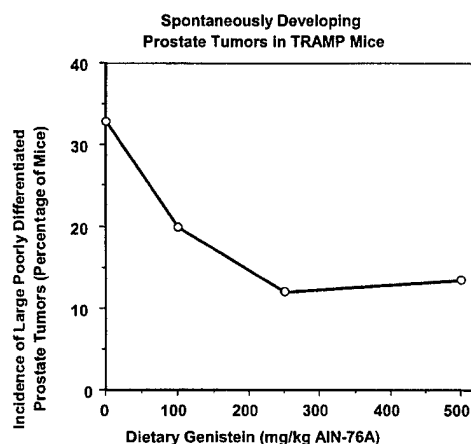
FIGURE 7 Genistein reduces the incidence of mice with advanced prostate tumors. The urogenital tract collected at necropsy was prepared for pathological evaluation of the prostate by established criteria (49,50). The results are the percentage of mice in each group with prostates displaying poorly differentiated adenocarcinomas; χ^2 test revealed that the frequency of transgenic mouse prostate adenocarcinoma (TRAMP) mice with poorly differentiated adenocarcinomas decreased significantly as a function of genistein in the diet ($P = 0.041$). [Data from (51). Permission granted from American Association for Cancer Research.]

diet devoid of genistein (C. A. Lamartiniere and J. Wang, unpublished data, 2001). ER-β and transforming growth factor-α mRNA were not altered by genistein. We speculate that genistein down-regulates expression of specific proteins and regulates cell proliferation and prostate cancer development. Should this down-regulation be extended to these sex steroid receptor and growth factor ligand and receptor proteins, this could provide a biochemical mechanism for the suppression of prostate cancer by genistein. Most interesting is that ER-β was not modulated by genistein. Not only does genistein bind with a greater affinity to ER-β than to ER-α (55), but the two ER have been shown to signal in different ways depending on ligand and response element. Also, ER-β is more involved in cell differentiation and ER-α is more involved in cell proliferation (56). Selective actions by genistein could explain both prostate gland differentiation via ER-β activation and reduced cell proliferation via down-regulated ER-α expression.

Genistein and prostate cancer summary

We demonstrated, in two animal models, that dietary physiological amounts of genistein can protect against chemically induced and spontaneously developing prostate cancers (47,51). We presented evidence that dietary genistein regulates, with specificity, sex steroid receptor and growth factor ligand and receptor mRNA expression. We speculate that these gene products contribute to chemoprevention of prostate cancer by genistein. Because postpubertal genistein exposure protects against prostate cancer development and can regulate sex steroid and growth factor signaling in animal models, we believe that genistein (or soy) can protect against prostate cancer in men.

DISCUSSION

We have demonstrated that the primary isoflavone component of soy, genistein, can protect against mammary (28–31) and prostate (47,51) cancers in rodent models. For mammary cancer protection, genistein exposure must first occur early in postnatal life. The importance of early mammary gland differentiation and carcinogenesis probably lies in pubertal development and estrogen surge, leading to oxidative DNA damage and cancer initiation. If the epithelial cells of the terminal end buds are differentiated, they are less susceptible for cancer (36,37). Also, we have demonstrated that early exposure to genistein exerts an imprinting-like effect on EGF receptor expression (38), a signaling pathway that plays a significant role in cell proliferation and cancer ontogeny. Imprinting is considered to set the pattern of gene expression early in development from which the adult responds; the pattern includes regulation of steroid receptor mechanisms and signal transduction. Consistent with this is the present demonstration that offspring imprinted early in life gain additional breast cancer protection with adult dietary exposure. Furthermore, we speculate that mammary cancer chemoprevention via cell differentiation and imprinting is not restricted to genistein. We believe that women imprinted by other means, for example, pregnancy or other nutritional differentiating chemicals, could benefit from ingesting soy as adults.

In reference to prostate cancer, we demonstrated that dietary genistein initiated at puberty suppressed spontaneously developing prostate cancer in transgenic mice (51). Short-term feeding of genistein from the pubertal to young adult period was able to down-regulate specific sex steroid receptor and growth factor ligand and receptor mRNA expression. We demonstrated that it is not necessary to give pharmacologic

concentrations of genistein to get beneficial effects. TRAMP mice fed genistein had serum genistein concentrations (125–400 nmol/L) (51) comparable with blood genistein concentrations of Asians eating a traditional diet high in soy (276 nmol/L) (45,46). This supports our earlier report that dietary physiological amounts of genistein could regulate biochemical actions in the prostate, that is, EGF receptor expression in rats (57).

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Genistein action in the prepubertal mammary gland in a chemoprevention model

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A diet high in soy is associated with many health benefits, including reduced incidence of breast cancer. The soy phytoestrogen, genistein, is hypothesized to contribute to mammary chemoprevention via interaction with estrogen receptors (ERs) alpha and/or beta. These steroid signaling pathways are believed to exert control over proliferation and differentiation of the mammary gland by a complex bidirectional interaction with the epidermal growth factor (EGF) signaling pathway. The current work was designed to study the role of these two pathways in prepubertal mammary gland growth. Female Sprague–Dawley CD rats were injected with genistein (500 μg/g body wt) or estradiol benzoate (EB) (500 ng/g body wt) on days 16, 18 and 20. Whole mount analysis of mammary glands from 21-day-old rats showed that both treatments resulted in significantly increased terminal end buds (TEBs), and increased ductal branching, compared with animals given the vehicle, dimethylsulfoxide (DMSO). Both effects were inhibited by blockage of ER function by pre-treating with 2 mg ICI 182,780/kg body wt, a steroidal anti-estrogen. Immunoblotting analyis of mammary gland extracts demonstrated increased epidermal growth factor receptor (EGFR) and progesterone receptor (PR) expression following treatment with EB or genistein. Tyrosine-phosphorylated EGFR, as measured by immunoprecipitation/immunoblotting was also increased, but when normalized to total receptors, there was no net effect. The expression and phosphorylation of downstream targets of the EGFR, mitogen activating kinase kinase (MEK 1 and 2) and extracellular signal regulated kinases 1 and 2 (ERK 1 and 2) were not significantly affected. Anti-estrogen pre-treatment prevented the increase in EGFR, phospho-EGFR and PR. The data indicate an ER-based mechanism of action for genistein in mammary gland proliferation and differentiation, which can lead to protection against mammary cancer.

Introduction

Asian women have a lower incidence of breast cancer than Western women (1,2). A major factor, which is thought to

contribute to this protection is the Asian diet, which is rich in soy products. Of the components of soy, the phytoestrogen, genistein, is the most studied. Our laboratory has shown that exposure of female rats to genistein either by injection (3) or via the diet (4) prior to puberty resulted in protection from dimethylbenz[a]anthracene-induced mammary carcinogenesis. The protection was derived from an early proliferative phase of mammary gland growth during the prepubertal period, resulting in an increase in TEBs (3), the most immature terminal ductal structures (5,6). The mitogenic effects of genistein treatment led to differentiation of the terminal end buds (TEBs) into lobules later in life (3); the latter are the most resistant terminal ductal structures to chemical carcinogenesis, due to their high degree of differentiation (5,6). The chemopreventative effect of accelerated glandular differentiation by genistein is similar to the protection derived from pregnancy early in life in humans (7), whereby hormonal exposure results in glandular maturity.

Of the multitude of biological effects identified for genistein, an important molecular basis for its action is binding to estrogen receptors (ER) alpha and ER beta (reviewed in ref. 8), a property that is conferred by its structural similarity to estradiol 17-beta. Another important action of genistein, which may contribute to its chemopreventative properties, is inhibition of the epidermal growth factor receptor (EGFR) tyrosine kinase (9). The EGF signaling cascade is important in the development of the rodent mammary gland (reviewed in ref. 10). Previous work using a chemoprevention protocol indicated that the expression of the EGFR and its ligand, TGF-alpha, were initially increased in the terminal ductal structures of the mammary glands of genistein-treated rats (11).

In ER-positive mammary cancer cells, treatment with estradiol induced synthesis of the EGFR mRNA and protein, an effect that was blocked in the presence of an anti-estrogen (12). In mice, treatment with estradiol increased tyrosine-phosphorylated EGFR in the mammary gland (13), suggesting a link between ERs and EGF signaling. Genistein was hypothesized to alter the EGFR tyrosine kinase activity by direct binding in multiple sites of the reaction (9), making it possible for growth regulation through non-ER-dependent mechanisms. Therefore, it is possible that the mechanism of action for genistein and estrogen in the growth of the pre-pubertal mammary gland may differ. The aim of the present research was to compare the mechanism of action of genistein and estradiol benzoate (EB) and to determine the role of ERs in the regulation of EGFR expression and mammary gland growth, which leads to differentiation.

Materials and methods

Animal treatment

offspring/dam at parturition; at this time standard lab chow was replaced with AIN-76A pellets (Harlan Teklad), which are devoid of phytoestrogens. Female offspring (ovary-intact) were assigned to the following groups: vehicle (dimethylsulfoxide) (DMSO) (Sigma, St Louis, MO); genistein (98.5% purity; Hoffmann-LaRoche, Basel, Switzerland); EB (Sigma); ICI 182,780 (Zeneca Pharmaceuticals, Cheshire, UK); genistein with ICI 182,780 pre-treatment; and EB with ICI 182,780 pre-treatment. On postnatal days 16, 18 and 20, female offspring were injected s.c. with 500 µg genistein/g body wt, 500 ng EB/g body wt or an equivalent volume of the vehicle. The treatment protocol for genistein is one that resulted in significant chemoprevention of chemically induced mammary tumors (3). An additional study using this protocol was done on prepubertally ovariectomized rats. On postnatal day 15, female rats were anesthetized using ketamine (10 mg/100 g)/xylazine (1.5 mg/100 g) and bilaterally ovariectomized. Injections of genistein, EB and vehicle were given on days 16, 18 and 20; rats were killed on day 21.

For animals with ICI 182,780 pre-treatment, 2.0 mg ICI 182,780/kg body wt was injected 30 min prior to genistein or EB treatments. Animals not receiving ICI 182,780 pre-treatment were injected with the vehicle. An additional dose of ICI 182,780 or vehicle was given on days 17 and 19. Rats were killed on postnatal day 21, 18–20 h after the last injection.

Evaluation of apoptosis

To ensure that the selected ICI 182,780 dose was growth-inhibitory without excessively triggering cell death, mammary glands from ovary-intact vehicle-treated and ICI 182,780-treated rats were analyzed for the presence of apoptotic cells, using six animals per group. The fourth abdominal mammary glands from vehicle- and anti-estrogen-treated animals were fixed overnight in 10% neutral-buffered formalin and embedded in paraffin blocks. Sections were placed onto glass slides and placed in xylene to remove paraffin, re-hydrated in a series of alcohols with increasing water content and immersed in Tris-buffered saline. DNA strand breakage was detected by labeling of the 3′-OH ends with biotinylated deoxynucleotides, catalyzed by terminal deoxynucleotidyl transferase (TdT-FragEL, Oncogene Research Products, Boston, MA), according to the manufacturer's instructions. Apoptotic nuclei were stained brown with 3,3′-diaminobenzidine and normal cell nuclei stained with methyl green counter stain. A positive control slide was treated with DNase (Promega, Madison, WI); negative control tissue received no TdT enzyme. Apoptotic and normal nuclei were enumerated in all epithelial cells using light microscopy; the number of apoptotic nuclei per section was expressed as a percentage of total cells. The median percentage of apoptotic nuclei for the two groups was compared using Mann–Whitney Rank Sum test (Sigma Stat., Jandel Scientific, San Rafael, CA).

Expression and localization of ER alpha by immunohistochemistry

Mammary glands of ovary-intact animals treated with genistein, EB or vehicle were analyzed for ER alpha expression. Because of the low expression of ER alpha, and its predominant epithelial expression in the rat mammary gland (14), immunohistochemistry (IHC) analyses were performed instead of immunoblotting. Also, this allowed for the analysis of cell-specific differences in expression. Fourth abdominal mammary glands were fixed in 10% neutral-buffered formalin overnight and embedded in paraffin blocks. Paraffin sections were cut with a microtome (5 µ thick) and placed on glass slides. Sections were stained for ER alpha with an antibody to the N-terminus (Zymed, San Francisco, CA) by an automated immunohistochemistry system (Ventanna Medical Systems, Tuscon, AZ), as described previously (15). Uterine and spleen tissue were used as positive and negative controls, respectively. A section of mammary gland from each animal was run in parallel without primary antibody, also serving as a negative control. Antibodies to the N-terminus are advantageous in IHC because of the lack of homology between ER alpha and ER beta in this region (16). Also, N-terminal antibodies are less susceptible to decreased epitope exposure following hormone binding than those directed to the hormone binding domain or the hinge region (17). Using light microscopy, each individual structure within a section was classified as a TEB, duct or lobule and analyzed for the percentage of positively stained cells and the average staining intensity. As the terminal end proximal to the nipple of all ducts are not seen when a mammary gland is cross-sectioned, and therefore could actually be a TEB, we referred to them as 'ducts', and not 'terminal ducts'. A scale from 1 (least intense) to 4 (most intense) was used to estimate intensity as described previously (11,18). For each animal, an average was calculated for intensity and percent of positive cells for TEB, ducts and lobules. The average values for each treatment group (*n* = 10/group) were compared using one-way analysis of variance (Sigma Stat, Jandel Scientific).

Immunoblotting analyses

EGFR signaling proteins and progesterone receptor

A more detailed description of the protocols may be found in our publications (11,15,19). Mammary glands were frozen in liquid nitrogen immediately after

dissection. The tissue was homogenized in lysis buffer containing 0.1% Triton X-100 with protease inhibitors, and the protein quantified on the extracts using a BCA assay (Pierce, Rockford, IL). The extracts were boiled in sample buffer containing 10% beta-mercaptoethanol and 10% SDS, and equal quantities of protein were separated by electrophoresis using SDS–polyacrylamide gels. Proteins were electroblotted onto nitrocellulose membranes and non-specific antibody binding was prevented by blocking with 5% non-fat dry milk. The following primary antibodies were used: 1:1000 dilution of anti-EGFR (Calbiochem, La Jolla, CA), 1:250 anti-progesterone receptor (PR) (Neomarkers/Labvision, Fremont, CA), 1:1000 anti-mitogen activating kinase kinase (MEK) 1/2 (Cell Signaling, Beverly, MA), 1:500 anti-phospho-MEK 1/2 (Cell Signaling), anti-extracellular signal regulated kinases (ERK) 1/2 (Cell Signaling) and anti-phospho-ERK 1/2. After incubation with HRP-conjugated secondary antibody, bands were detected using chemiluminescence (Pierce) and exposed to X-ray radiography film. Band intensity was quantified using scanning densitometry. Values were compared using one-way ANOVA (Sigma Stat, Jandel Scientific).

Cytokeratins

Mammary glands from ovary-intact rats were crushed under liquid nitrogen and homogenized in buffer containing 2% SDS, 5 mM beta-mercaptoethanol and protease inhibitors as described above. Samples were centrifuged at 12 000 r.p.m. to remove cellular debris and fat. Total protein was determined using a Bradford-based assay (Bio-Rad, Hercules, CA) using BSA as a standard. Equal amounts (20 µg) of total protein were separated by electrophoresis and transferred as described above. Membranes were incubated with a 1:750 dilution of monoclonal anti-pan cytokeratin (Sigma), which recognizes cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19. The antibody does not react with non-epithelial tissue and, therefore, rat skeletal muscle was used as a negative control. Statistical comparison of band intensity was achieved as described above.

Quantification of phosphorylated EGFR

Mammary gland extracts from ovary-intact and ovariectomized rats were prepared as described above. Immunoprecipitation of tyrosine-phosphorylated proteins was achieved by incubating equal amounts of protein with anti-phosphotyrosine antibody (Signal Transduction Laboratories, Carpinteria, CA) for 30 min at 4°C while rotating. Protein A–agarose beads were added (Calbiochem) and the samples rotated for an additional 3 h. The immunocomplexes were pelleted by centrifugation and washed four times with lysis buffer (described above), resuspended in loading buffer containing 5% SDS, 0.5 mM Tris and 5% beta-mercaptoethanol, boiled for 5 min and subjected to SDS–PAGE western blotting as described above. Membranes were blotted with a 1:1000 dilution of anti-EGFR (Calbiochem).

Mammary gland whole mounts

Whole mounts from ovary-intact animals were prepared as described previously (20,21). Briefly, mammary glands were fixed in 10% neutral-buffered formalin, defatted in acetone, re-hydrated, stained in alum carmine, dehydrated in a series of graded alcohols, cleared in xylene and coverslipped with mounting media. Light microscopy (200× magnification) was used to classify and quantify the terminal ductal structures within an area 1.5 mm from the edge of the gland, distal to the nipple as described previously (3,11). Criteria used for the classification of the terminal ductal structures as TEBs, terminal ducts (TDs) and lobules I are based on those described elsewhere (5,6). For quantitative branching analysis, mammary glands were photographed using a digital camera (Polaroid DMCIe) connected to a dissecting microscope (Leica MZ6). Measurements of ductal length and total gland area were taken using Scion Images Software (Scion, Frederick, MD, through the NIH). For assessment of the degree of side branching, each gland was divided into thirds, and one branch per area was selected. Starting at the portion of the gland most distal to the nipple, each branch was traced to the primary duct. The number of branches, including secondary branches, lateral buds and lobules were counted. The length of each branch was recorded, and the ratio of the total number of branch points per unit length (arbitrary units) was calculated. The average of the three values was taken for each gland (*n* = 4/group), and the mean values for each group were compared with controls using a one-way ANOVA. Pair-wise multiple comparisons were made using Tukey test (Jandel Scientific).

Results

Immunolocalization of ER alpha by IHC

Expression of ER alpha was localized mainly in the nuclei of the mammary epithelium; few positive stromal cells were present. The average percentage of positively stained cells in the TEBs, ducts and lobules of genistein- or EB-treated animals
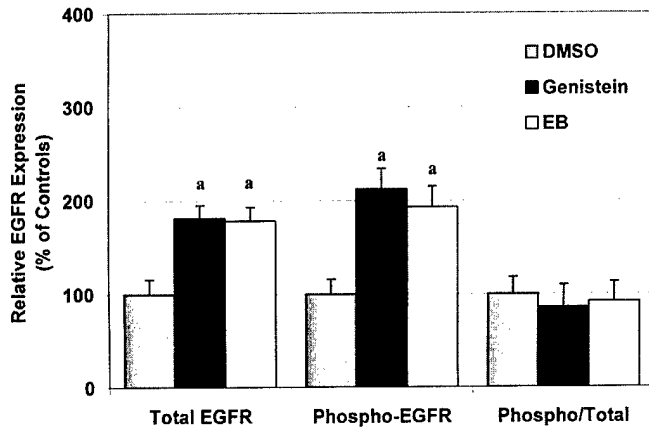
Fig. 1. Measurement of total and tyrosine-phosphorylated EGFR from mammary gland extracts from ovary-intact 21-day-old rats. Total EGFR was measured by western blot analysis using anti-EGFR. Tyrosine-phosphorylated EGFR was immunoprecipitated with anti-phosphotyrosine and immunoblotted with anti-EGFR. The percentage of phosphorylated EGFR was calculated by dividing phosphorylated into total EGFR. Densitometric values were reported as a percentage of the controls ± SEM. [a]$P < 0.001$, compared with controls.

Fig. 2. Measurement of total and tyrosine-phosphorylated EGFR from mammary gland extracts from ovariectomized 21-day-old rats. Total EGFR were measured by western blot analysis using anti-EGFR. Tyrosine-phosphorylated EGFR were immunoprecipitated with anti-phosphotyrosine and immunoblotted with anti-EGFR. The percentage of phosphorylated EGFR was calculated by dividing phosphorylated into total EGFR. Densitometric values were reported as a percentage of the controls ± SEM. [a]$P < 0.05$, compared with controls.

Table I. ER alpha immunohistochemical analysis in mammary ductal structures

Structure	DMSO	Genistein	EB
Average % positive cells			
TEBs	49 ± 1	51 ± 5	43 ± 7
Ducts	59 ± 2	59 ± 5	42 ± 5
Lobules I	55 ± 3	52 ± 7	41 ± 6
Average staining intensity			
TEBs	3.2 ± 0.2	2.0 ± 0.2[a]	1.7 ± 0.2[a]
Ducts	3.4 ± 0.3	2.5 ± 0.1[a]	2.3 ± 0.4[a]
Lobules I	3.1 ± 0.2	2.5 ± 0.2	2.3 ± 0.3

[a]$P < 0.05$, compared with controls using one-way ANOVA; $n = 8–10$/group.

Fig. 3. Uterine to body weight ratio in ovary-intact rats treated ± genistein, EB and/or ICI 182,780 (ICI). Uterine wet weights (mg) were divided by body weights (g) and the result multiplied by a factor of 100. [a]$P < 0.001$, when compared with vehicle-treated rats.

was not significantly different from controls (Table I). Average staining intensity was significantly decreased in the TEBs and ducts of genistein- and EB-treated rats, while staining intensity was slightly decreased in the lobules.

Analysis of EGFR and downstream kinases by western blotting

Evaluation of mammary gland extracts for proteins involved in EGF signaling revealed that genistein and EB treatments significantly increased expression of total EGFR and phosphorylated EGFR compared with controls (Figure 1). Normalization of the band intensity of phospho-EGFR to total EGFR for each sample resulted in no significant difference compared with controls. Expression of the downstream kinases MEK 1/2 and ERK 1 and 2 were not significantly altered by genistein or EB; analysis using phospho-specific antibodies revealed no significant difference, compared with controls (data not shown).

As circulating levels of estradiol are increased by injections of genistein in this model (15), we evaluated EGFR expression in the mammary gland of ovariectomized prepubertal rats to rule out indirect action of genistein via estradiol. Expression of EGFR and tyrosine-phosphorylated EGFR was increased in the mammary gland of EB- and genistein-treated ovariectomized rats (Figure 2). As seen in ovary-intact rats, there was no net effect on the ratio of phosphorylated to total EGFR. As similar results were obtained in ovariectomized and ovary-
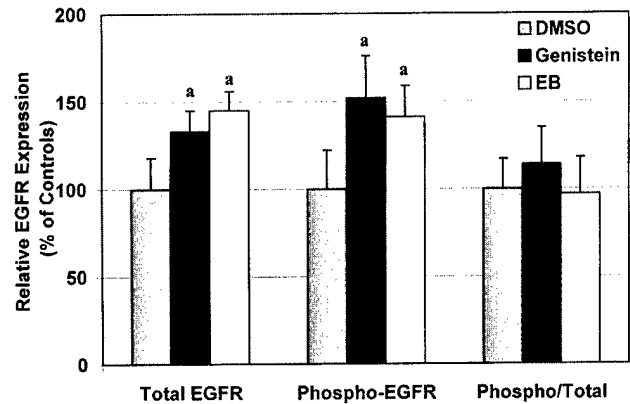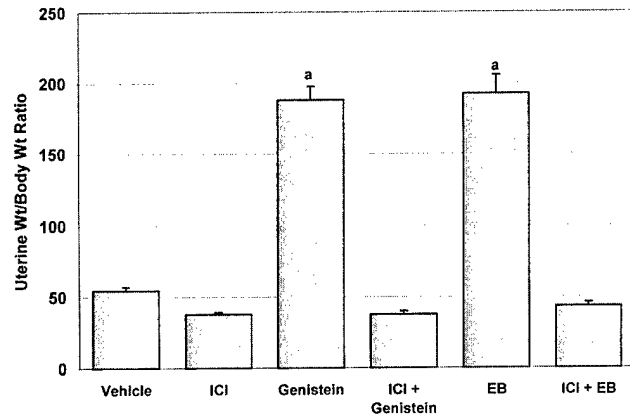
intact rats, analysis of downstream kinases was restricted to ovary-intact rats only.

Cytokeratin expression

As western blotting analyses are reflective of the amount of target protein per total protein, an increase in the number of epithelial structures in the mammary gland after treatment with genistein or EB could result in a greater percentage of epithelial protein loaded onto the gel, compared with control animals. This would result in falsely increased EGFR and PR, which are expressed highly in the epithelium. Therefore, epithelial-specific markers were used to rule out a potential artifact. There was no significant difference in the expression of cytokeratins following genistein or EB treatment, compared with controls (data not shown). There were no cytokeratins in the skeletal muscle.

Evaluation of ICI 182,780 dose

Uterine wet weight was used as an indicator of anti-estrogenic activity by ICI 182,780. Genistein and EB significantly increased uterine wet weight in ovary-intact rats (Figure 3). The increase was blocked when 2.0 mg ICI 182,780/kg body wt was given prior to either compound; ICI 182,780 alone
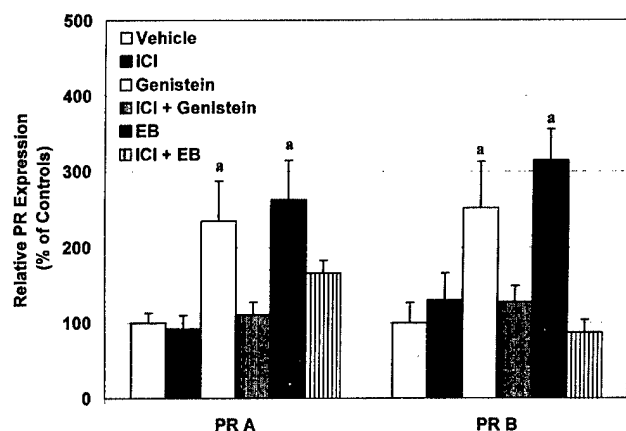
Fig. 4. Immunoblotting analysis for PR (A and B isoforms) from mammary gland extracts from ovary-intact rats treated ± genistein, EB and/or ICI 182,780 (ICI) (n = 8/group). Densitometric values were reported as a percentage of the controls ± SEM. [a]P < 0.05, compared with controls.

Fig. 5. Immunoblotting analysis for EGFR (phosphorylated and dephosphorylated forms) from mammary gland extracts from ovary-intact rats treated ± genistein, EB and/or ICI 182,780 (ICI) (n = 8/group). Densitometric values were reported as a percentage of the controls ± SEM. [a]P < 0.05, compared with controls.

induced a slight decrease in uterine weights, when compared with vehicle-treated rats. However, the change was not statistically significant. There were no significant alterations in body weight in ICI 182,780-treated rats, compared with controls (data not shown).

Evaluation of apoptosis

Apoptotic nuclei were enumerated and compared in mammary glands from ovary-intact vehicle- and ICI 182,780-treated animals to determine if the anti-estrogenic action of ICI 182,780 resulted in cell death. DNase-treated positive control tissue exhibited brown staining in the nuclei. The negative control, which lacked the enzyme required to label fragmented DNA, showed no positive staining. A small percentage of apoptotic nuclei were observed in the ductal epithelium and the TEBs. The mean percentage of apoptotic cells in mammary glands from the vehicle- and ICI 182,780-treated animals were 0.35 ± 0.10 and 0.25 ± 0.06%, respectively. Statistical comparison of the median values (0.34% for controls and 0.22% for ICI 182,780-treated animals) did not indicate a significant difference Mann–Whitney Rank Sum test (Sigma Stat, Jandel Scientific).

Effect of anti-estrogen pre-treatment on EGFR and PR expression

To provide biochemical evidence of inhibition of the ER function by ICI 182,780, measurement of an estrogen-responsive protein, the progesterone receptor, was performed on mammary extracts by immunoblotting analysis. Genistein or EB injections resulted in significantly increased PR (A and B isoforms) protein expression in ovary-intact rats (Figure 4) compared with the vehicle. In ovariectomized rats, the effect on PR expression was similar to ovary intact rats. PR isoform A expression was 150 and 165% for genistein- and EB-treated rats, respectively, when normalized to control mean (data not shown). PR B expression for genistein- and EB-treated mammary glands was 170 and 161% of controls (data not shown). The increase in PR isoforms in the mammary glands of ovary-intact rats was inhibited by pre-treatment with ICI 182,780. ICI 182,780 alone had no significant effect on PR expression.

The increase in EGFR expression by genistein and EB in ovary-intact females was inhibited by pre-treatment with ICI 182,780 (Figure 5). The anti-estrogen alone had no significant
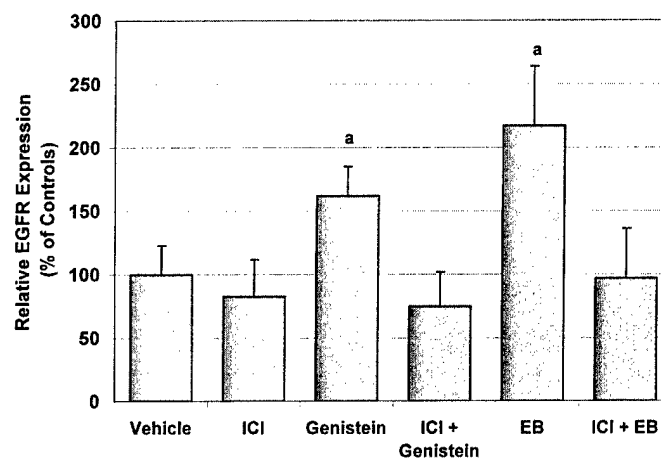
Table II. Quantification of mammary terminal ductal structures of rats treated ± genistein, EB and/or ICI 182,780 (ICI)

Treatment	TEBs	TD	Lobules I
Vehicle	34 ± 3	48 ± 5	1 ± 1
ICI	6 ± 3[a]	40 ± 6	1 ± 1
Genistein	72 ± 6[a]	32 ± 4	1 ± 1
ICI + genistein	8 ± 4[a,b]	50 ± 8	1 ± 1
EB	66 ± 8[a]	29 ± 4[a]	3 ± 2
ICI + EB	10 ± 3[a,c]	54 ± 4	1 ± 1

[a]P < 0.05, compared with vehicle-treated animals using one-way ANOVA.
[b]P < 0.001, compared with genistein-treated rats using multiple pairwise comparison (Tukey test).
[c]P < 0.001, compared with EB-treated rats using multiple pairwise comparison (Tukey test).

effect on EGFR expression when compared with vehicle-treated animals. There was no significant effect on the amount of tyrosine-phosphorylated EGFR following treatment with ICI 182,780, ICI 182,780 + genistein or ICI 182,780 + EB, when compared with vehicle-treated rats (data not shown).

Whole mount analysis

Analysis of mammary terminal ductal structures indicated approximately twice the number of TEBs in genistein- or EB-treated rats, compared with the vehicle (Table II). Pre-treatment with ICI 182,780 inhibited this effect, resulting in approximately two-thirds fewer TEBs than vehicle-treated animals. The number of TEBs in mammary glands from animals treated with ICI 182,780 + genistein or ICI 182,780 + EB were significantly fewer than those of animals treated with genistein or EB alone, respectively. Animals given ICI 182,780 alone had significantly fewer TEBs when compared with vehicle-treated rats. Only EB treatment altered the number of TDs, resulting in a significant decrease (40% fewer). When ICI 182,780 was given alone or prior to genistein or EB, the number of TDs was not significantly different from controls. There were no significant effects on the number of lobules I in any treatment group.

Figure 6 depicts mammary gland whole mounts representative of each treatment. The mammary glands from animals treated with the anti-estrogen were minimally branched, with
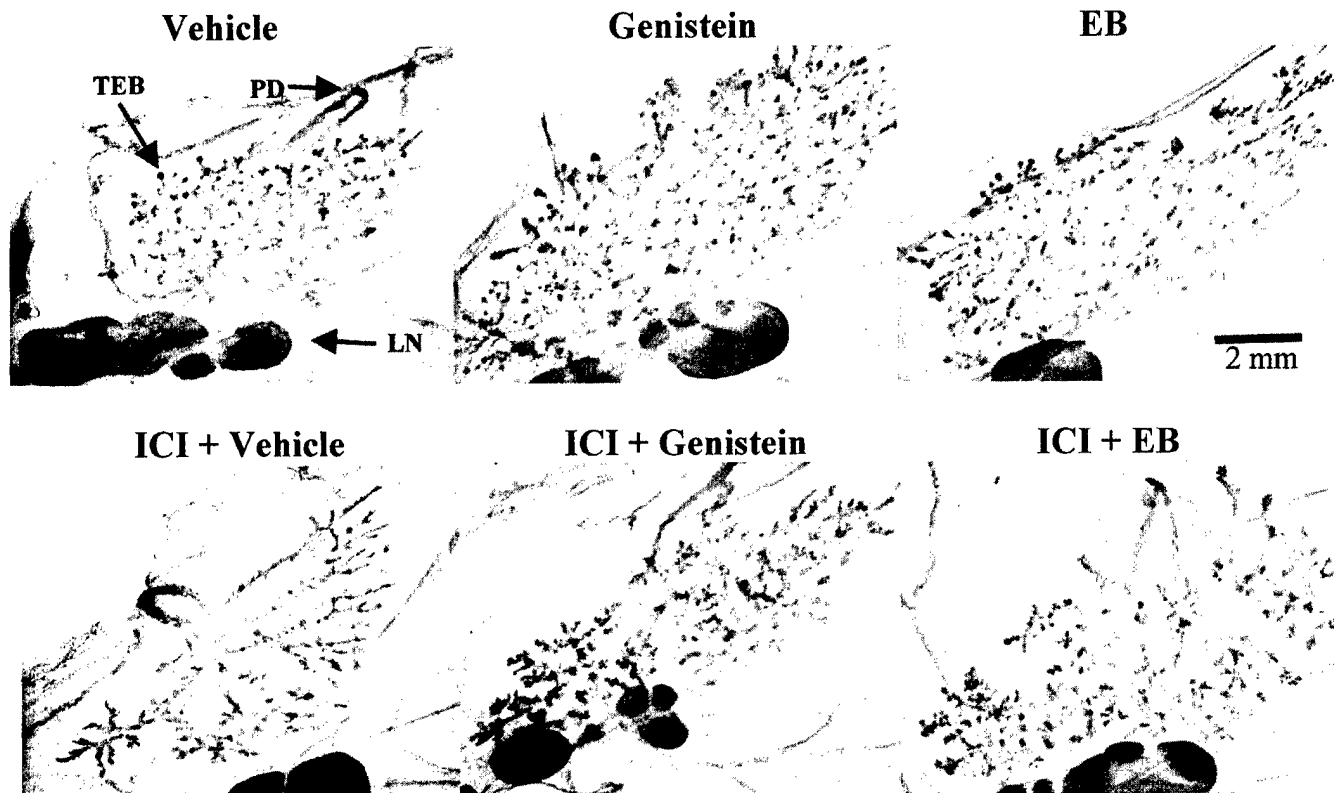
Fig. 6. Representative mammary gland whole mounts taken from ovary-intact rats treated ± genistein, EB and/or ICI 182,780 (ICI). Photographs were taken at 2× magnification. TEB = terminal end bud, LN = lymph node, PD = primary duct, which leads to the nipple.

very few tertiary branches and small lateral buds. The branching density of mammary glands of vehicle-treated rats was greater than those of anti-estrogen-treated rats, with many secondary/tertiary branches and alveolar units. Glands from animals treated with genistein or EB were more extensively branched than controls, with numerous alveolar units present. The interior branching of glands of animals treated with ICI 182,780 + EB and ICI 182,780 + genistein appeared less dense than controls, but not as sparse as animals given ICI 182,780 alone. Glands from animals treated with ICI 182,780 + genistein and ICI 182,780 + EB were composed mainly of short branches and lateral buds, while control glands were composed primarily of longer branches and alveolar units.

In order to provide quantitative data to support our visual observations, the numbers of branch points along three random ducts per gland were counted. The degree of branching in the EB- and genistein-treated rats was significantly greater than that of controls and animals pre-treated with ICI 182,780 (Figure 7). When ICI 182,780 was given prior to genistein or EB, the degree of branching was similar to controls. ICI 182,780 alone significantly decreased the degree of side branching by 30%, compared with controls.
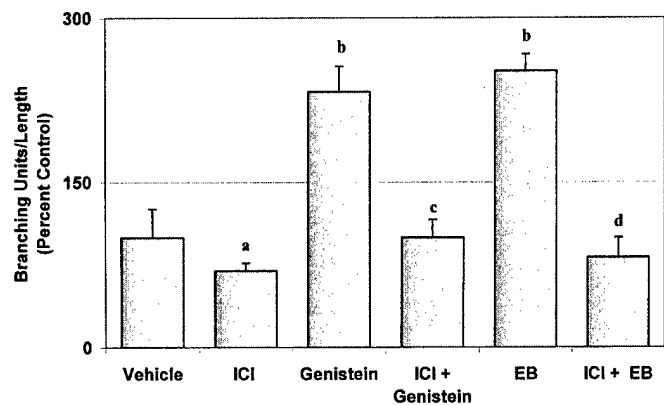

Fig. 7. Analysis of branching units per length (arbitrary units) from digital photographs of mammary gland whole mounts taken from ovary-intact rats treated ± genistein, EB and/or ICI 182,780 (ICI). Computerized measurements were taken of the length of three branches per gland ($n = 4$ animals/group), from the main lactiferous duct to the distal terminal structure. All side structures, including secondary/tertiary ductal branches, lateral buds, and alveolar units were counted. The average number of side structures was divided by the length of the duct. [a]$P < 0.05$, [b]$P < 0.001$, compared with vehicle-treated rats (one-way ANOVA); [c]$P < 0.001$, compared with genistein treated rats (Tukey test); [d]$P < 0.001$, compared with EB-treated rats (Tukey test).

Discussion

Decreased expression of ER alpha in response to genistein has been observed in ER-positive tissues, including the uterus (15) and prostate (22). Similarly, others have shown that estrogen treatment reduced the expression of ER alpha in breast cancer cells (23–25). In the mammary gland, semi-quantitative immunohistochemical analysis showed decreased ER alpha staining intensity in the TEBs and ducts following treatment with genistein and EB. The decrease in staining intensity may

indicate receptor down-regulation, which is hypothesized to occur as part of the cyclic nature of ER expression. While the significance of the down-regulation and cycling of ERs is not clear, it may represent a mechanism of regulatory control of estrogen responsiveness of the tissue. The induction of the estrogen-inducible protein, PR, by genistein and EB suggests that decreased immunoreactivity of ER correlated with activation of the receptor. Likewise, the down-regulation of ER may

be explained on the basis of PR to repress ER transcriptional activity (26). Decreased expression of ER alpha may also reflect proliferation, as proliferating epithelial cells of the mammary glands of young virgin rats do not express the receptor (14,27). This is consistent with our previous results, which indicated that prepubertal genistein treatment resulted in a proliferative mammary gland at day 21, which led to a more differentiated structure evident at day 50 (3). These actions resulted in subsequent down-regulated EGFR expression (11) and reduced cell proliferation (3) in the TEBs of adult rats. The similar decrease in immunoreactivity between genistein and EB suggests a common molecular action.

However, *in vitro* studies suggest that the molecular action of phytoestrogens may differ from estradiol. The binding affinity of genistein for ER beta is greater than that for ER alpha, while estradiol-17-beta binds to both receptors with similar affinity (28,29). Recent work has shown that genistein is more capable of regulating transcriptional activity of ER beta than ER alpha, while estradiol appears to be equally effective on both receptors (30). Therefore, genistein may exert control of ER-dependent gene expression primarily through ER beta. Despite the preference of genistein for ER beta, ER alpha may play a larger role in mammary gland development, as the structure and function of the mammary gland in the ER beta knockout mouse appears to be normal (31), while mammary gland development is severely impaired in ER alpha knockout mice (32,33). Interestingly, the expression of ER beta in the mammary glands of prepubertal rats is greater than that of ER alpha (27), but the exact function of ER beta in mammary development remains to be elucidated.

Immunoblotting analyses for EGFR indicated an increase in the expression of receptors after injection with genistein and EB, again indicating a similarity in the mechanism of action between the two compounds. These results are in agreement with data reported previously indicating increased EGFR in the mammary glands of genistein-treated adult rats (11). As EGFR and PR up-regulation also occurred in the mammary glands of ovariectomized, pre-pubertal rats treated with either genistein or EB, an indirect action via increased circulating estradiol following genistein treatment (15) can be ruled out. Alternatively, the possibility of stimulation of local estrogen production in the mammary gland by genistein cannot be excluded. There was no net effect on tyrosine-phosphorylation of the receptors, making a direct action on the EGFR by genistein unlikely. A similar observation was reported where dietary genistein decreased prostatic EGFR and tyrosine-phosphorylated EGFR (19), indicating no net effect on phosphorylation. Therefore, it is not likely that growth enhancement through direct interaction with the EGFR by genistein is occurring. Rather, genistein is acting in a manner similar to EB, and the increase in EGFR is a consequence of ER activation. This conclusion is further supported by the ablation of this response in the presence of the ICI anti-estrogen. Similar results were shown by Ankrapp *et al.* (34), who reported the inhibition of EGF-induced stimulation of TEB growth in anti-estrogen-treated prepubertal mice. Collectively, the data support a requirement for ERs in EGF signaling in the rat mammary gland.

Stimulation of the EGFR signaling cascade following ligand binding, receptor dimerization and autophosphorylation involves the phosphorylation of downstream kinases, including Raf, MEK and ERK (reviewed in refs 35,36). Therefore, increased autophosphorylation should correlate with increased

receptor tyrosine kinase activity, resulting in increased phosphorylation of downstream targets. The lack of an observable effect on the expression and/or phosphorylation of MEKs1/2 and ERKs 1/2 may simply be explained by the absence of any net effect on autophosphorylation. Alternatively, a single time point following multiple injections was chosen to measure expression and may not reflect overall fluctuations in phosphorylation and dephosphorylation. Differential cell-specific effects may not be detected by immunoblotting analysis of proteins extracted from the entire gland, which may be affected by a larger percentage of epithelial-derived protein in the treated animals, due to epithelial proliferation. This was not an issue for the EGFR, which is highly expressed in the epithelium (11), as indicated by the lack of a treatment-related increase in cytokeratins.

The analysis of mammary gland morphology in genistein and EB-treated prepubertal rats indicated a growth-stimulatory response, manifested as an increase in TEBs and ductal branching. Increased TEBs in the area of the gland closest to the lymph node following prepubertal genistein treatment has been reported previously by our laboratory (3,11). These proliferative effects were inhibited by pre-treatment with ICI 182,780, suggesting that the main effect of the anti-estrogen was to block ERs in the TEBs, which are located in the most proliferative area of the gland, most distal to the nipple (for review, see ref. 37). TEBs also express a greater percentage of ER alpha (5,14) and ER beta (27) compared with the more differentiated structures. Thus, TEBs may be more sensitive than more differentiated structures to the anti-estrogenic action of ICI 182,780, which binds to both receptors without transcriptional activation (38). The atrophied appearance of the mammary glands of animals treated with ICI 182,780 + vehicle suggests that the actions of endogenous estrogens, detectable in vehicle-treated 21-day-old rats (15), were blocked. However, we cannot rule out the possibility that alterations to other hormone pathways (androgen, progesterone, prolactin) may have contributed to glandular atrophy.

Glandular proliferation was not completely inhibited when ICI 182,780 was given prior to genistein or EB. The branching of the mammary glands of these animals was more dense than that of animals treated with ICI 182,780 alone, but not as dense as that of control animals. This effect was attributed to the presence of lobuloalveolar structures and longer branches in the glands of the controls, while the majority of branch points in animals given ICI 182,780 + genistein or ICI 182,780 + EB were shorter branches or lateral buds. As the anti-proliferative action of ICI 182,780 in the mammary gland is reversible if the treatment is discontinued (39), genistein or EB may stimulate the growth in areas of the gland other than that closest to the lymph node after the effects of ICI 182,780 have begun to subside. Therefore, ICI 182,780 treatments were given on days 16–20. When ICI 182,780 treatments were given only on days 16, 18 and 20, no inhibition of genistein- or EB-induced mammary gland growth, PR or EGFR expression was observed. Alternatively, other hormones such as prolactin or progesterone may be responsible for ductal growth and branching occurring within the interior of the gland.

Growth inhibition by ICI 182,780 is not likely to occur through interaction with stromal ER alpha, as Russo *et al.* (14) have reported ER alpha to be absent in the mammary stroma of rats, and our data show low stromal expression of ER alpha. The minimal expression of stromal ER alpha in rats is in contrast to tissue recombination studies in mice, which

demonstrated that stromal, not epithelial ER alpha, was responsible for estrogen-induced epithelial growth through a paracrine mechanism (40). ER beta is expressed in some stromal cells of the rat mammary gland (27), but its function is unknown.

A low percentage of apoptotic nuclei were observed in the mammary glands of both control- and anti-estrogen-treated animals, suggesting that ICI 182,780 achieved growth inhibition by blocking estrogen action, not by inducing cell death. This is in agreement with Wakeling et al. (41), who reported that ICI 182,780, when given at concentrations that resulted in anti-estrogenicity in the absence of toxicity, induced a cytostatic response in MCF-7 cells. Others have shown that implantation of ICI 182,780 pellets directly into the mammary glands of mice resulted in local inhibition of growth, while the contralateral glands were unaffected (39). The data suggested a direct and critical role for local estrogen on ERs in mammary development. The inability of genistein to stimulate mammary gland growth in the presence of the anti-estrogen again supports an estrogen-like, ER-based mechanism, and not direct action on the EGFR.

The pharmacologic model used in the current study involved a short duration of exposure to genistein, a treatment that results in chemoprevention (3). Previous studies have shown that short exposure to estrogenic compounds during development also resulted in chemoprevention of spontaneously originating (42) and chemically induced mammary tumors (43,44). The data are in contrast to the estrogen-dependency of mammary cancer with respect to the processes of initiation, promotion and progression (reviewed in ref. 45). Thus, both timing and duration of exposure play a role in chemoprevention.

In conclusion, genistein and EB act via the ERs to regulate PR and EGFR in the rat mammary gland. Genistein up-regulates EGFR expression, but not phosphorylation of the receptor. Genistein action in the prepubertal mammary gland accounts for initial proliferative action that results in gland differentiation and protection against mammary cancer (3,4).

Acknowledgements

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